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Research Paper

Surface Characteristics of Spacecraft Components Affect the Aggregation of Microorganisms and May Lead to Different Survival Rates of Bacteria on Mars Landers

ANDREW C. SCHUERGER,[1] JEFFREY T. RICHARDS,[2] PAUL E. HINTZE,[3] and ROGER G. KERN[4]

ABSTRACT

Layers of dormant endospores of *Bacillus subtilis* HA101 were applied to eight different spacecraft materials and exposed to martian conditions of low pressure (8.5 mbar), low temperature (−10°C), and high CO_2 gas composition and irradiated with a Mars-normal ultraviolet (UV–visible–near-infrared spectrum. Bacterial layers were exposed to either 1 min or 1 h of Mars-normal UV irradiation, which simulated clear-sky conditions on equatorial Mars (0.1 *tau*). When exposed to 1 min of Mars UV irradiation, the numbers of viable endospores of *B. subtilis* were reduced three to four orders of magnitude for two brands of aluminum (Al), stainless steel, chemfilm-treated Al, clear-anodized Al, and black-anodized Al coupons. In contrast, bacterial survival was reduced only one to two orders of magnitude for endospores on the non-metal materials astroquartz and graphite composite when bacterial endospores were exposed to 1 min of Mars UV irradiation. When bacterial monolayers were exposed to 1 h of Mars UV irradiation, no viable bacteria were recovered from the six metal coupons listed above. In contrast, bacterial survival was reduced only two to three orders of magnitude for spore layers on astroquartz and graphite composite exposed to 1 h of Mars UV irradiation. Scanning electron microscopy images of the bacterial monolayers on all eight spacecraft materials revealed that endospores of *B. subtilis* formed large aggregates of multilayered spores on astroquartz and graphite composite, but not on the other six spacecraft materials. It is likely that the formation of multilayered aggregates of endospores on astroquartz and graphite composite is responsible for the enhanced survival of bacterial cells on these materials. Key Words: Mars—Astrobiology—Planetary protection—Spacecraft materials. Astrobiology 5, 545–559.

INTRODUCTION

MICROBIAL CONTAMINATION of robotic spacecraft is composed of a diversity of non–spore-forming, spore-forming, and non-culturable microorganisms that could pose a significant risk to near-term Mars surface missions by increasing the forward contamination of scientific payloads,

[1]University of Florida and [2]Dynamac Corporation, Space Life Sciences Lab; and [3]Corrosion Technology Testbed, Kennedy Space Center, Florida.
[4]Jet Propulsion Lab, Mars Exploration Directorate, Pasadena, California.
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local landing sites, or the global martian environment. To model the forward contamination of Mars, the microbial ecologies of spacecraft must be understood from initial assembly of spacecraft components through the operational termination of each mission. Robotic landers or rovers sent to Mars undergo significant levels of spacecraft cleaning and sterilization activities to reduce the bioloads prior to launch to less than 3×10^5 total viable spore-forming bacteria per vehicle for non-life detection missions (Barengoltz, 1997). However, the total microbial bioloads on spacecraft, including spore-formers, non–spore-formers, and non-culturable species, likely will be one to two orders of magnitude higher than this level based on models for the launched bioloads of unmanned robotic landers (Dillon *et al*., 1973) and recent work with detecting non-culturable microorganisms on spacecraft (Venkateswaran *et al*., 2001, 2003). Although there probably will be significant losses of species diversity and viable bioloads during the 6–8-month cruise phase to Mars (Schuerger, 2004), it is possible that viable terrestrial microorganisms have and will be successfully landed on the martian surface.

The conditions on Mars are only slightly better for the survival of terrestrial microorganisms than the conditions found in interplanetary space (Schuerger, 2004). The key biocidal factors for terrestrial microorganisms on Mars are: (i) low atmospheric pressure, (ii) high solar ultraviolet (UV) irradiation, (iii) severe desiccating conditions, (iv) extreme temperature fluctuations, (v) solar particle events, and (vi) galactic cosmic rays (reviewed by Schuerger, 2004). Despite these extreme conditions many studies have demonstrated that terrestrial microorganisms can survive simulated martian conditions as long as they are protected from solar UV irradiation (Packer *et al*., 1963; Hagen *et al*., 1964, 1967; Hawrylewicz *et al*., 1964; Green *et al*., 1971; Foster *et al*., 1978; Koike *et al*., 1996; Mancinelli and Klovstad, 2000; Schuerger *et al*., 2003; reviewed by Schuerger, 2004).

Most of the early literature on microbial survival under martian conditions mixed viable cells of test organisms into terrestrial or Mars analog soils (Packer *et al*., 1963; Hagen *et al*., 1964, 1967; Hawrylewicz *et al*., 1964; Scher *et al*., 1964; Foster *et al*., 1978). In addition, microbial species during martian simulations have been placed on agar surfaces (Imshenetsky *et al*., 1973), in crushed sandstone (Fulton, 1960; Roberts and Wayne, 1962), in limonite (Green *et al*., 1971), on glass (Imshenetsky *et al*., 1967) or stainless steel (Green *et al*., 1971) planchets, and on aluminum coupons (Hagen *et al*., 1971; Koike *et al*., 1996; Mancinelli and Klovstad, 2000; Schuerger *et al*., 2003; Schuerger and Kern, 2004). Although these studies examined different questions concerning microbial survival under simulated martian conditions, a few general conclusions may be drawn from this body of work. First, dormant spores of terrestrial microorganisms survived well under low temperature, low pressure, and N_2 or CO_2 atmospheres, though reductions in microbial populations of one to several orders of magnitude occurred (Packer *et al*., 1963; Hagen *et al*., 1964; Hawrylewicz *et al*., 1964; Green *et al*., 1971; Imshenetsky *et al*., 1973; Foster *et al*., 1978). Second, UV irradiation was the key parameter that determined survivability of microorganisms under simulated martian conditions; direct exposure to UV irradiation resulted in rapid and nearly complete inactivation of microbial cultures (Packer *et al*., 1963; Green *et al*., 1971; Koike *et al*., 1996; Mancinelli and Klovstad, 2000; Schuerger *et al*., 2003). Third, thin contiguous layers of Mars analog soils measuring from several tens to several hundred micrometers thick were generally adequate for protecting microorganisms from the lethal effects of UV irradiation (Packer *et al*., 1963; Mancinelli and Klovstad, 2000; Schuerger *et al*., 2003). However, small individual dust particles measuring up to 50 μm in diameter were unable to protect endospores of *Bacillus subtilis* from Mars UV fluence rates (Schuerger *et al*., 2003). Fourth, freeze–thaw cycles generally did not reduce microbial survival rates under simulated martian conditions (Packer *et al*., 1963; Young *et al*., 1964; Foster *et al*., 1978).

In contrast, what is notably lacking in this body of literature is a comparative study to determine whether diverse spacecraft materials can affect the survival of terrestrial microorganisms under martian conditions. One key question that must be addressed with regard to predicting the risks of forward contamination of Mars by spacecraft microbial contaminants is whether terrestrial microbes are able to survive, grow, and replicate on the surfaces of spacecraft materials. If terrestrial microorganisms on spacecraft survive and replicate on Mars, then their presence might affect the success of future life-detection experiments. Furthermore, the effects of spacecraft materials on the survival of terrestrial microorganisms under

martian conditions will impact the selection of proper sanitation and sterilization protocols used during spacecraft assembly.

The primary objectives of the current study were: (a) to determine whether endospores of a common spacecraft contaminant, *B. subtilis,* will adhere to a diversity of spacecraft materials at different rates, (b) to determine whether spore layers on eight spacecraft materials are inactivated at the same rates under Mars simulations in which normal UV fluence rates are generated in combination with low pressure, low temperature, and high CO_2 atmospheres, and (c) to examine surface characteristics of spacecraft materials and the aggregation of endospores of *B. subtilis* on these spacecraft materials to determine whether endospores of *B. subtilis* formed multilayered colonies on specific materials (see also Schuerger and Kern, 2004). *B. subtilis* was chosen for this study because it is a common contaminant of spacecraft surfaces (Puleo *et al*., 1973, 1977; Venkateswaran *et al*., 2001; La Duc *et al*., 2003, 2004), and it has been used in previous studies under martian conditions (Hagen *et al*., 1964; Hawrylewicz *et al*., 1964; Green *et al*., 1971; Koike *et al*., 1996; Mancinelli and Klovstad, 2000; Schuerger *et al*., 2003).

MATERIALS AND METHODS

Mars simulation chamber

Simulated martian experiments were conducted within the Mars Electrostatic Chamber (MEC) operated by the Electrostatics & Surface Physics Lab in the Operation & Checkout Building at the Kennedy Space Center, FL. The MEC was described previously as the Mars Simulation Chamber by Schuerger *et al*. (2003). In brief, the MEC is a stainless steel low-pressure cylindrical chamber that measures 1.5 m long by 0.8 m in diameter and is capable of accurately simulating martian temperature (−100 to +25°C), gas composition (pure CO_2), pressure (4–12 mbar), and UV irradiation. Temperature control of spacecraft materials was achieved with a liquid nitrogen thermal control system (model TP2555, Sigma Systems Corp., San Diego, CA) placed within the MEC. Atmospheric composition was created by flushing the MEC with gas delivered from commercially obtained tank mixes of pure CO_2 (>99.99% purity) (Boggs Gases, Titusville, FL). An automated pressure control system was built into the MEC and permitted accurate control of experimental pressure setpoints, pump-down rates, and back-to-air repressurization rates.

The Mars-normal UV irradiation was created by two 450-W xenon-arc lamps (lamp model 6262, Oriel Instruments, Stratford, CT) mounted outside the MEC (Schuerger *et al*., 2003). The UV-enriched light was distributed within the MEC by a series of UV-transmitting fiber optic bundles (Optran UVNS non-solarizing fibers, Ceram-Optec, East Longmeadow, MA). In addition to UV irradiation, the xenon-arc lamps supplied visible (VIS) (400–700 nm) and infrared (700–1,100 nm) photons at fluence rates of 218 and 222 W m^{-2}, respectively. The MEC lighting system was calibrated to deliver 5.3, 7.6, 33.1, and 46 W m^{-2} of UVC, UVB, UVA, and total UV (200–400 nm), respectively to the upper surfaces of spacecraft materials. The UV-VIS-infrared fluence rates were created to simulate an optical depth (*tau*) of 0.1 on equatorial Mars at its mean orbital distance from the Sun (312×10^6 km; 1.5236915 AU) and were based on previous models of martian surface irradiation (see Kuhn and Atreya, 1979; Appelbaum and Flood, 1990; Cockell *et al*., 2000; Patel *et al*., 2002; Schuerger *et al*., 2003). An optical depth of 0.1 *tau* on Mars was used to represent a worst-case scenario of UV irradiation that might be encountered under extremely clear-sky conditions.

Spacecraft materials

Eight spacecraft materials were used in these experiments, which included two brands of uncoated aluminum (model M4985, Seton, Inc., Branford, CT; and aluminum-6061), a graphite/polycyanate composite material (M55J/BtCy-1, hereafter called graphite composite), a quartz/polycyanate composite material (AQ II/EX1515, hereafter called astroquartz), chemfilm (also called alodine)-treated aluminum, clear-anodized aluminum, black-anodized aluminum, and 304 stainless steel. Prior to use, the spacecraft materials were placed in 70% ethanol, sonicated for 5 min, rinsed in sterile deionized water (SDIW), air-dried in a laminar-flow hood, and then UV-sterilized by exposing both sides of each coupon for 1 h to a Hg-lamp (254 nm) at an intensity of 6.5 W m^{-2}. Following sterilization, the spacecraft materials were placed inside sterile 25-ml screw-top centrifuge tubes and stored at 25°C. The six

metal coupons were composed of generally smooth surfaces with randomly scattered shallow cracks and pits. The smoothest metal surfaces were observed on the two uncoated aluminum coupons, and pits were observed on all metals. The stainless steel and chemfilm-treated aluminum surfaces possessed networks of shallow cracks, but the cracks in the stainless steel coupons were slightly deeper (2–4 μm) than were observed in chemfilm-treated aluminum (1–2 μm). Small pits in the five types of aluminum coupons were generally 1–6 μm in depth. The surfaces of the astroquartz and graphite composite materials were very smooth with no pits or cracks observed in any of the coupons examined.

Microbiological techniques

Endospores of *B. subtilis* HA101 were grown in a liquid sporulation medium, washed, and concentrated according to the procedures of Mancinelli and Klovstad (2000) and Schuerger *et al*. (2003). Layers of *B. subtilis* spores were applied to spacecraft materials by depositing 2.4×10^6 viable endospores, suspended in 100-μl microdrops of SDIW, to the upper surfaces of individual 1-cm^2 coupons. Microdrops of suspended endospores of *B. subtilis* were dried on spacecraft materials at 25°C overnight in a microbial incubator. Schuerger *et al*. (2003) determined that this drying process had no adverse effects on the viability of *B. subtilis* endospores. Layers of attached endospores were microscopically inspected prior to use with a high-resolution video microscope (model VH-7000, Keyence Corp. of America, Woodcliff Lake, NJ). Endospores on the six metal spacecraft surfaces were observed as uniform monolayers with randomly dispersed individual endospores. In contrast, endospores on the astroquartz and graphite composite materials formed large multilayered aggregates of spores intermixed with randomly dispersed individual cells. The deposition process produced consistent results for each spacecraft material, and all coupons were used as prepared.

After layers of *B. subtilis* HA101 were prepared on all eight spacecraft materials, coupons were assayed for the numbers of viable endospores recoverable with a Most Probable Numbers (MPN) assay procedure to determine whether the endospores adhered differentially to the eight spacecraft materials. Each coupon was processed separately using a MPN procedure described previously (Mancinelli and Klovstad, 2000; Schuerger *et al*., 2003). In brief, each coupon was placed in a separate 50-ml centrifuge tube with 20 ml of SDIW, vortex-mixed for 2 min, serially diluted, and 20 μl of each dilution pipetted into 16 individual wells of a 96-well plate each containing 180 μl of a *Bacillus* spore medium (*i.e*., six dilutions dispensed). A MPN code was recorded for each assay, and the numbers of viable endospores recovered from spacecraft components were estimated with the probability tables published by Koch (1994).

In a second series of tests, spore layers on all eight spacecraft materials were exposed to martian conditions of pressure (8.5 mbar), temperature (−10°C), pure CO_2 atmosphere (>99.99%), and irradiated with a Mars-normal UV-VIS-near-infrared (NIR) spectrum. Spore layers on spacecraft materials were exposed to 1 min or 1 h of Mars-normal UV irradiation, which simulated clear-sky conditions on equatorial Mars (0.1 *tau*) at the mean orbital distance to the Sun. Mars simulations lasted 4 h total elapsed time from initial evacuation of room air from the MEC to repressurization of the chamber. After UV exposure, the coupons were processed separately using the MPN procedure described above. Internal controls were designed into the UV irradiation experiments to separate the effects of the UV irradiation from the other effects of holding endospores of *B. subtilis* at the simulated martian conditions. These controls consisted of set of spore-coated coupons from all eight spacecraft materials maintained inside the MEC system but shielded from UV irradiation, and a second set of controls of all eight materials outside the MEC system maintained at Earth-normal conditions of 1,013 mbar, 25°C, and a normal O_2/N_2 terrestrial atmosphere.

Scanning electron microscopy (SEM)

Bacterial layers on all eight spacecraft materials were prepared for SEM to determine the distribution and layering of endospores on each material. Spore layers were prepared and dried at 25°C for 24 h, as described above, and inspected with a high-resolution video imaging system (model VH-7000, Keyence Corp. of America). Only spore layers on the various spacecraft materials that exhibited similar spore-density and spore-dispersal characteristics as the coupons used in the MEC experiments were processed for

SEM. Endospores were not fixed, but were coated with gold and then imaged immediately with a model LEO1455 scanning electron microscope (Leo Electron Microscopy, Inc., Thornwood, NY).

Advancing and receding contact angles for tests of hydrophobicity

The hydrophobicity of a surface may be estimated by measurement of the water contact angles to that surface (Adamson, 1990). The contact angle is the angle between the surface of a given material and a water droplet on that surface. A large contact angle (*i.e.*, significantly greater than 90°) indicates that a surface is hydrophobic. Two contact angles, advancing and receding, were measured for each material. The advancing contact angle is the angle measured when the water is expanding over the surface, while the receding contact angle is measured when the water recedes from the surface. Both angles are necessary to characterize the hydrophobicity of a surface; however, the receding angle more closely models what occurs when a droplet evaporates on a surface. Contact angle measurements were made with a Cahn Instruments Inc. (Cerritos, CA) model DCA-312 dynamic contact angle analyzer. The DCA-312 analyzer employs a Wilhelmy plate method in which a sample is slowly lowered into and then raised from a reservoir of deionized water (18 MΩ), which allowed the advancing and receding contact angles, respectively, to be measured. Three separate samples of each material were tested separately for three immersion cycles, which resulted in nine total measurements ($n = 9$). Advancing and receding contact angles were measured using cleaned spacecraft coupons in which endospores of *B. subtilis* were not present, and in which the coupons were air-dried between measurements for 3 min. The precision of measuring both the advancing and receding contact angles was ±1.4° for multiple measurements of the same coupon and ±6.2° for a set of three coupons per material. Thus, there was more variability among different samples of each spacecraft material than there was among measurements of the same sample.

Statistical procedures

Statistical analyses were conducted with version 8.0 of the PC-based Statistical Analysis System (SAS Institute, Inc., Cary, NC). For all experiments, 0.25 power transformations were used to induce homogeneity of variances of individual treatments; all data are presented as untransformed numbers. Transformed data were subjected to analysis of variance procedures (PROC GLM) followed by protected least-squares mean separation tests ($P \leq 0.05$). For the UV-irradiation experiments, the number of endospores per material that survived inside the MEC when exposed to UV irradiation (N) was divided by the number of endospores per material that survived in the non-UV irradiated controls (N_o) maintained within the MEC system. Thus the numbers in Fig. 2 correspond to log-reductions in surviving endospores of *B. subtilis* (N/N_o) that were exposed to UV irradiation under simulated martian conditions.

RESULTS

A comparison of the number of endospores recoverable by the MPN assay for each type of spacecraft material revealed differences in adherence between the metal versus non-metal surfaces (Fig. 1). Between 5% (Al-6061) and 12% (chemfilm-coated aluminum) of deposited endospores were recovered from the metal surface, whereas significantly higher numbers of endospores were recovered from astroquartz (48% recovered) and graphite composite (85% recovered) materials.

The number of surviving endospores recovered after exposure to martian conditions of low pressure (8.5 mbar), pure CO_2 atmosphere, low temperature (−10°C), and UV irradiation for 1 min or 1 h also differed between metal and non-metal surfaces (Fig. 2). Following a 1-min UV exposure, all metal coupons exhibited three to four orders of magnitude reduction in the numbers of viable endospores of *B. subtilis*. In contrast, astroquartz and graphite composite coupons exhibited only one to two orders of magnitude reduction in the numbers of recovered endospores. Furthermore, this trend was maintained following a 1-h UV exposure. No viable endospores were recovered from any of the metal surfaces after 1 h, whereas astroquartz and graphite composite exhibited only two to three orders of magnitude reduction in the numbers of recovered endospores. Thus, while the numbers of viable endospores on all metal surfaces recovered after 1 h of UV irradiation were reduced to levels below the detection limit of the MPN assay (<180 viable endospores

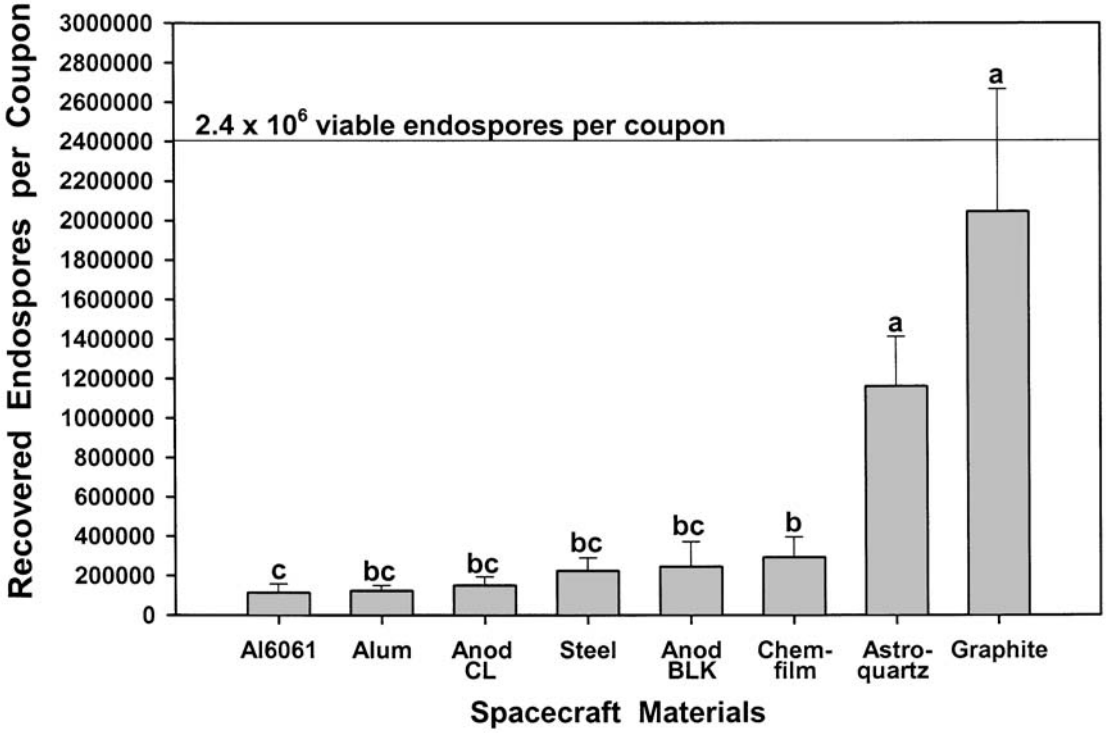

FIG. 1. Numbers of endospores of *B. subtilis* recovered from eight spacecraft materials. The mean initial density of endospores per coupon was 2.4×10^6 viable spores as estimated with the MPN procedure. Different letters indicate significant differences among treatments based on analysis of variance and protected least-squares mean separation tests ($P \leq 0.05$; $n = 6$). Al6061, aluminum-6061; Alum, aluminum; Anod CL, clear-anodized aluminum; Anod BLK, black-anodized aluminum.

per coupon), between 10^3 and 10^4 viable endospores were recovered from astroquartz and graphite composite coupons.

Internal and external controls were used to assess the effects of the simulated martian conditions with and without UV irradiation on the survival of *B. subtilis* endospores on each type of spacecraft material (Fig. 2). Furthermore, by presenting the data in Fig. 2 as log reductions of surviving endospores, based on the algorithm N/N_o, the reductions were equally scaled to each other and independent of the inherent adhesion of spores to the materials. Internal controls for the UV irradiation experiment indicated that there was no apparent effect of a 4-h exposure to simulated martian conditions ($P > 0.05$; $n = 6$). The non–UV-irradiated Earth and Mars controls were similar for all spacecraft materials tested ($P > 0.05$; $n = 6$).

SEM was used to examine the distribution of endospores on the various materials. Results indicated that propagules of *B. subtilis* were dispersed randomly as individual endospores on all metal coupons but frequently clumped into large multilayered aggregates on astroquartz and graphite composite coupons (Fig. 3). There were no large multilayered aggregates of endospores observed on any of the metal surfaces tested. However, small single-layered colonies composed of between a few to as many as a dozen endospores were observed periodically on the metal surfaces. Individual endospores of *B. subtilis* were often observed embedded in preexisting surficial cracks and pits (arrows, Fig. 3) on the metal surfaces. The deepest pits were observed on uncoated aluminum, and the most highly cracked surfaces were observed on chemfilm-treated aluminum and stainless steel. No pits or cracks were observed on the astroquartz and graphite composite materials. Shallow and wide depressions were commonly observed on the clear- and black-anodized aluminum coupons. In summary, the SEM investigation revealed that the spore layers occurred as monolayers of individual endospores on the metal surfaces and as multilayered aggregates of spores on the astroquartz and graphite composite materials.

During the above experiments, the microdrops

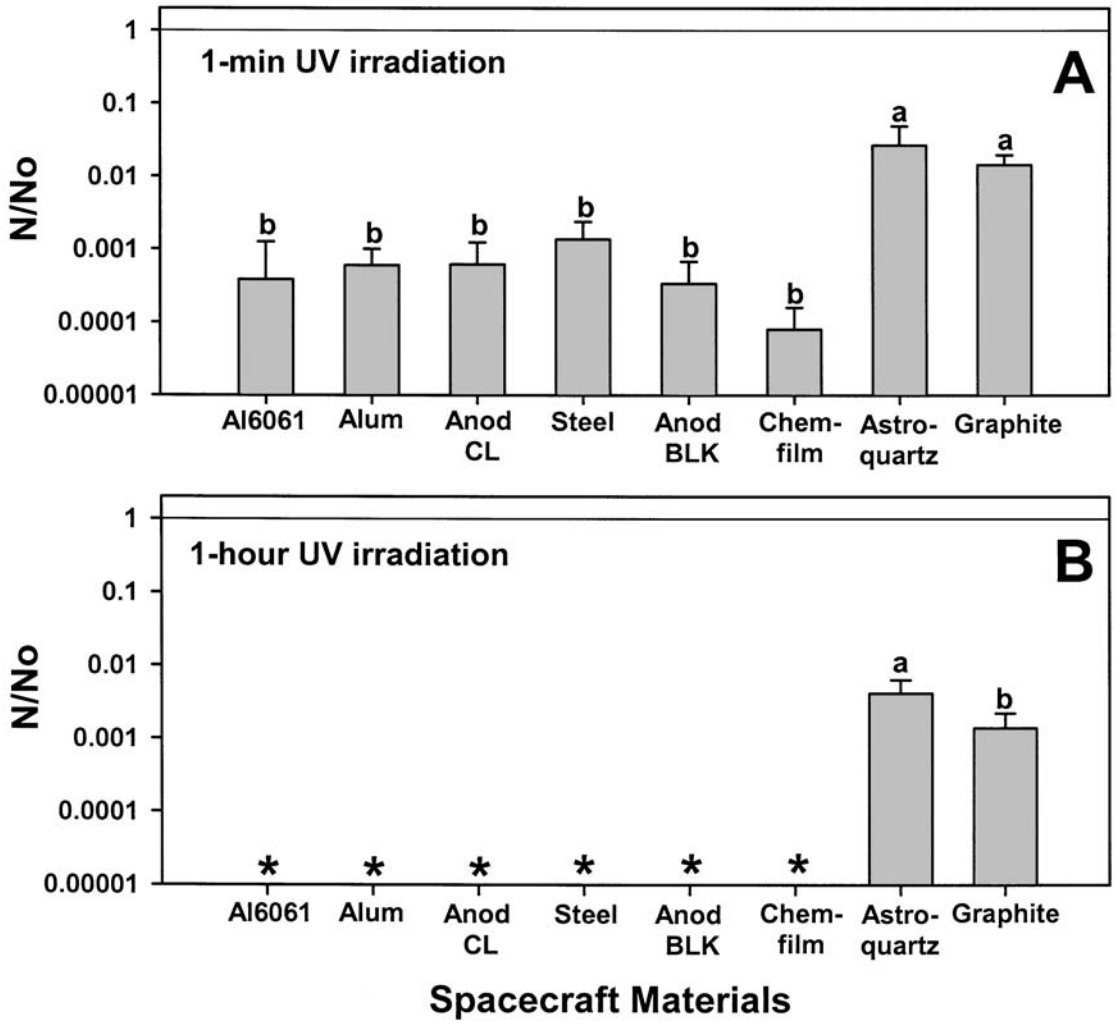

FIG. 2. Effects of UV irradiation under martian conditions on the numbers of surviving endospores of *B. subtilis* recovered from eight spacecraft materials. The mean initial density of endospores per coupon was 2.4×10^6 viable spores as estimated with the MPN procedure. Monolayers of endospores were UV irradiated for either 1 min (**A**) or 1 h (**B**) at 8.5 mbar, −10°C, and under pure CO_2 atmospheres. Different letters indicate significant differences among treatments based on analysis of variance and protected least-squares mean separation tests ($P \leq 0.05$; $n = 6$). Al6061, aluminum-6061; Alum, aluminum; Anod CL, clear-anodized aluminum; Anod BLK, black-anodized aluminum. *Below the limit of detection.

on all eight spacecraft materials were visually inspected as they dried onto the coupons to monitor the drying process of the spores onto the spacecraft materials. Results indicated that the drying process of the deionized water microdrops on the spacecraft materials progressed in basically two fashions, depending on the material. First, microdrops of deionized water on all metal surfaces followed a pattern of drying in which the water would evaporate without the diameters of the microdrops changing (Fig. 4A). In general, microdrops on the metal surfaces measured approximately 6–8 mm in diameter throughout the entire drying process. In contrast, microdrops of deionized water on both astroquartz and graphite composite coupons dried in a manner in which the outer edges of the microdrops contracted into smaller and smaller microdrops as the water evaporated (Fig. 4B). Microdrops on astroquartz and graphite composite started out with approximate diameters of 6–8 mm, but would contract to 2–3 mm in diameter after 24 h in the microbial incubators. Few endospores were observed on the outside of the dried edges of the contracted microdrops on astroquartz and graphite composite coupons after 24 h. These observations are consistent with the conclusion that the drying process on astroquartz

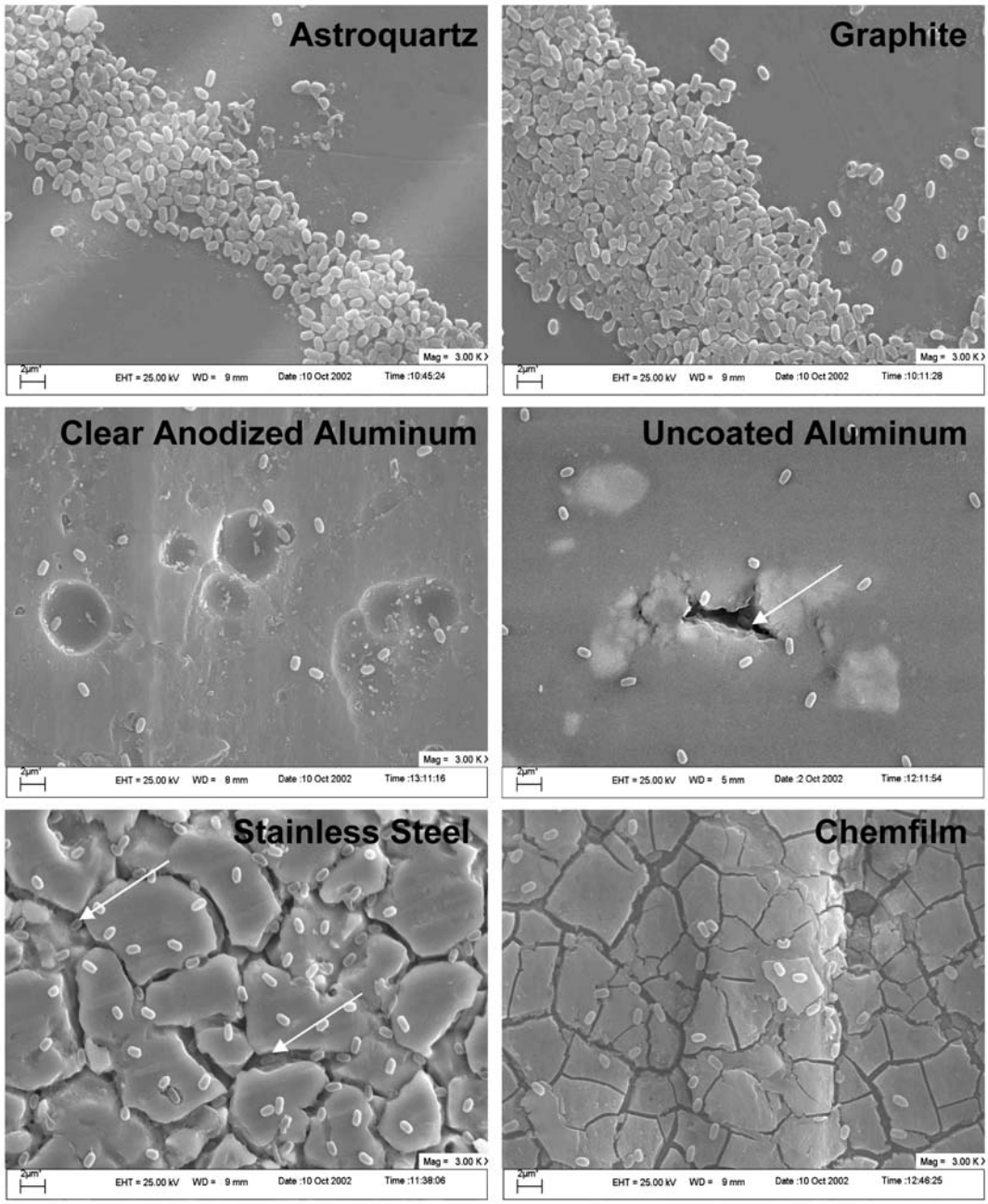

FIG. 3. SEM images of endospores of *B. subtilis* dispersed onto six of the eight spacecraft materials used in the current experiments. Endospores were observed as monolayers of individual cells on all metal surfaces (black anodized and aluminum-6061 not shown), but were observed to form multilayered aggregates on the non-metal surfaces of astroquartz and graphite composite. Endospores were commonly observed lodged within cracks, crevices, and pits on the various metals (arrows), but not on the smooth non-metal materials astroquartz and graphite composite.

and graphite composite coupons acted to concentrate the endospore suspensions into multilayered aggregates by the contraction of the outer edges of the microdrops. The endospores in the microdrops placed on the metal coupons failed to form multilayered aggregates because the outer edges of the drying microdrops did not change as the water evaporated, and, thus, the randomly dispersed endospores settled to the metal surfaces and dried in place. In addition, the metal coupons often exhibited changes in color or luster upon which the microdrops dried, but

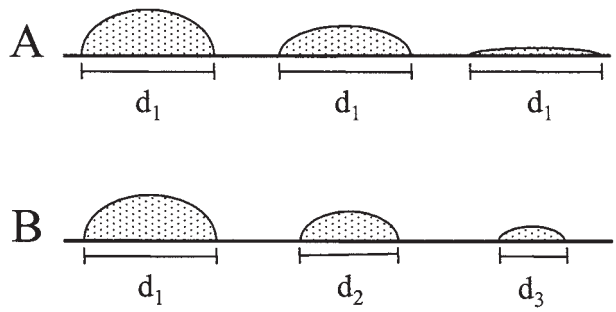

FIG. 4. Schematic drawing of the drying process of endospore-laden microdrops on metal surfaces (A) and the non-metal materials astroquartz and graphite composite (B). During the drying process on metals, the 100-μl microdrops dried evenly without a reduction in the diameters (d) of the microdrops. In contrast, during the drying process on the non-metal materials astroquartz and graphite composite, the diameters of the microdrops decreased with the evaporation of water and, thus, resulted in concentrating the endospores of *B. subtilis* into multilayered aggregates of spores.

both astroquartz and graphite composite coupons exhibited no visual changes in surface characteristics during the drying process.

Though some differences in advancing and receding contact angles were observed among individual spacecraft materials (Fig. 5), there was no clear pattern observed between metal and non-metal surfaces that might explain the results of the spore recovery (Fig. 1) and UV-irradiation (Fig. 2) experiments. Advancing and receding contact angles were similar among all eight spacecraft materials tested with the advancing contact angles being approximately twice the receding contact angles (Fig. 5). The average advancing and receding contact angles for metal surfaces were 83° and 32°, respectively. The average advancing and receding contact angles for non-metal surfaces were 83° and 27°, respectively. Thus, the overall averages of the advancing and receding contact angles for all spacecraft materials were 83° (range 61°–93°) and 30° (range 21°–46°), respectively. The differences in advancing and receding contact angles between the metal and non-metal surfaces were not significant ($P > 0.05$; $n = 9$).

A wide range of topographic features on the spacecraft materials were observed in which bacteria might become entrapped or buried during pre-launch processing. The topographic features observed during our SEM study were used to create a series of illustrations (Figs. 6 and 7) that depict the three-dimensional characteristics likely present on the spacecraft materials examined in the current study. These features included shallow pits (Fig. 6A), which were observed on clear-anodized and black-anodized aluminum (Fig. 3), and a range of features with increasing depth and

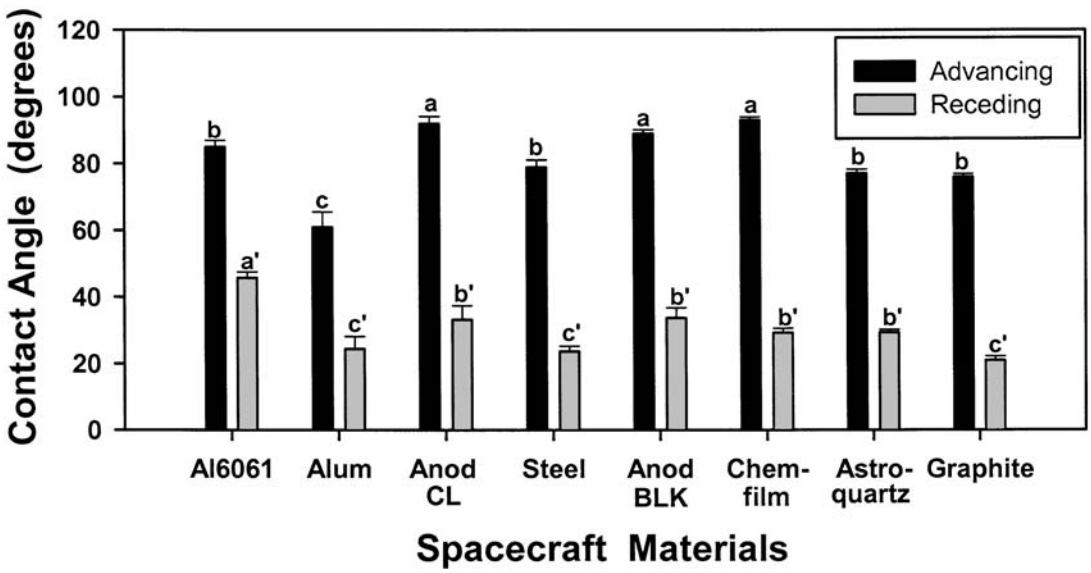

FIG. 5. Advancing and receding contact angles to measure the hydrophobicity of the eight spacecraft materials. Different letters indicate significant differences among treatments based on analysis of variance and protected least-squares mean separation tests ($P \leq 0.05$; $n = 9$). Non-primed letters are for advancing contact angles, and primed letters are for receding contact angles. Al6061, aluminum-6061; Alum, aluminum; Anod CL, clear-anodized aluminum; Anod BLK, black-anodized aluminum.

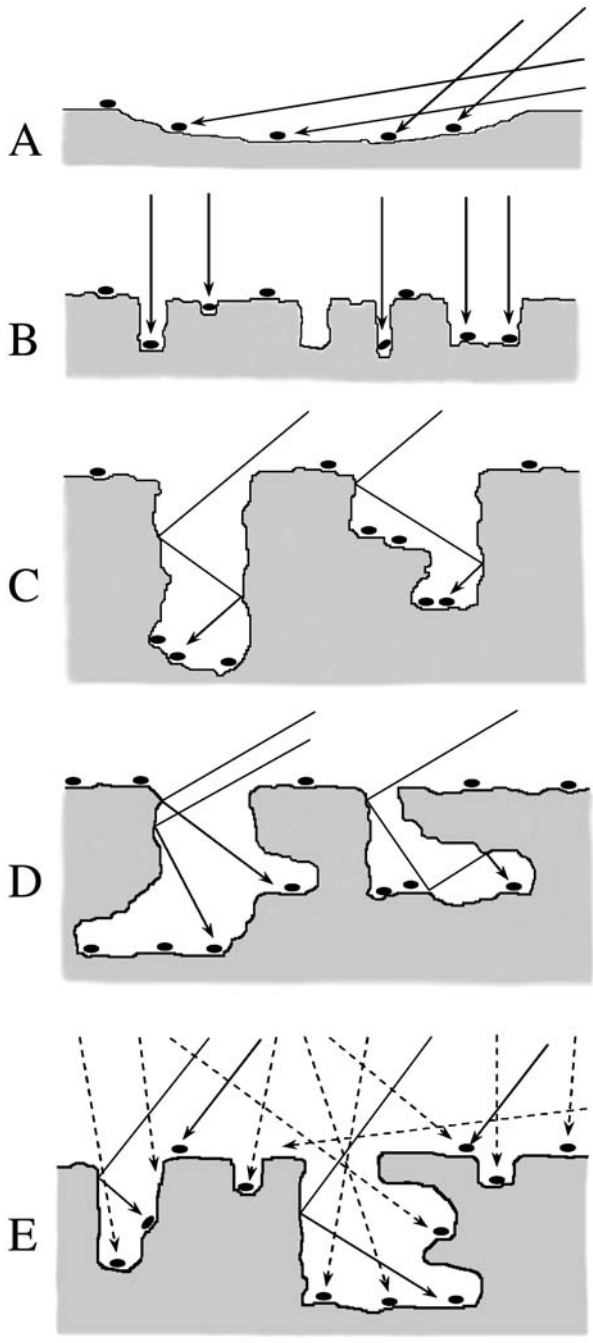

FIG. 6. A–E: Illustrations depicting the range of topological features observed on most metal surfaces with SEM. Solid arrows represent the direct beam UV irradiation, and the dashed arrows represent the diffuse beam UV irradiation impinging upon spacecraft materials on Mars. In general, UV photons can penetrate into the various cracks, crevices, and pits on the metal spacecraft materials if no UV-absorbing deposits occlude the openings.

complexity of cracks, crevices, and pits (Figs. 6 and 7), which were found on Al-6061, uncoated aluminum, stainless steel, and chemfilm-treated aluminum. Astroquartz and graphite composite materials proved to be extremely smooth in nature with few indentations in which bacteria might become lodged.

DISCUSSION

The surface characteristics of spacecraft materials affect the aggregation of bacterial spores in liquids by altering the drying processes on spacecraft components. This effect was most dramatic for the two non-metal materials used in the current study and may reflect a general difference between metal and non-metal spacecraft materials. Endospores of *B. subtilis* HA101 formed uniform monolayers of individual spores on metal surfaces, but generally collected into large multilayered aggregates on the non-metal materials astroquartz and graphite composite. The primary result of spore aggregation on astroquartz and graphite composite surfaces was to increase the

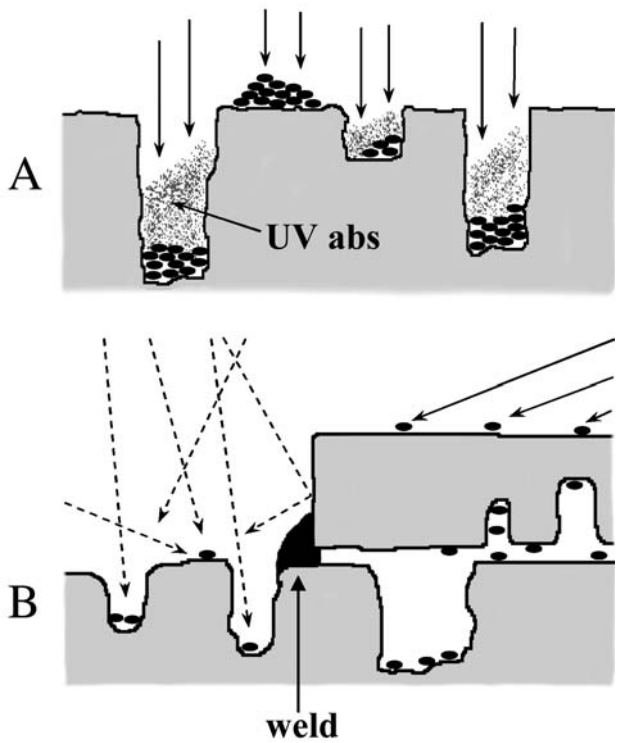

FIG. 7. Illustrations depicting the range of topological features observed on most metal surfaces with SEM. Solid arrows represent the direct beam UV irradiation, and the dashed arrows represent the diffuse beam UV irradiation impinging upon spacecraft materials on Mars. However, in these illustrations the attenuation of UV irradiation is achieved by UV absorbing (UV abs) materials (**A**), multilayered colonial aggregates (A), or a solid weld (**B**) between two spacecraft components.

survival of spores on these two materials under simulated martian conditions that included Mars-normal UV irradiation. This phenomenon is consistent with the results reported by Mancinelli and Klovstad (2000) in which multilayered colonies of *B. subtilis* HA101 were shown to enhance the survival of endospores under simulated martian UV irradiation. Spore survival can be enhanced in multilayered colonies by a process in which the overlying cells absorb UV photons and, thus, protect underlying cells against the biocidal effects of UV irradiation (Mancinelli and Klovstad, 2000). It seems likely then that this multilayering effect observed for bacterial endospores on astroquartz and graphite composite surfaces was responsible for enhancing the survival of endospores of *B. subtilis* on these two materials. In addition, the changes in color and luster on the metal coupons noted in the current study are interpreted to indicate that the deionized water might have penetrated and possibly altered the surface characteristics of the metal materials in such a way as to affect the drying process of water microdrops. Furthermore, there were numerous cracks, crevices, and pits observed on the upper surfaces of all six metal coupons, and these topological features may have increased the adhesive qualities of the metals, resulting in more uniform drying than was observed on astroquartz and graphite composite.

In a preliminary report, Schuerger and Kern (2004) interpreted the drying phenomenon as an indication that astroquartz and graphite composite surfaces were more hydrophobic than the surfaces of the six metals. However, new research reported herein on the advancing and receding contact angles of the eight spacecraft materials does not support the conclusion that astroquartz and graphite composite materials were more hydrophobic than the metals tested (Fig. 5). In fact, both astroquartz and graphite composite have advancing and receding contact angles below the average values of all samples (83° and 30°, respectively). Considering only the receding angles, which should closely resemble what occurs as water droplets evaporate, the diameters of water drops on aluminum-6061 would be expected to decrease as the water evaporated since aluminum-6061 had the largest receding contact angle. However, this was not observed, and, thus, the hydrophobicities of the spacecraft materials likely were not the sole cause of the observed drying trends. It is possible that the water droplets interacted with the surface of the metals in a manner that prevented the constriction of the droplet diameters during drying. These changes are currently not understood but may include the early formation of oxides or other corrosion products, absorption of water into the metals that alter the surface characteristics of the materials, or changes in the adhesion of water to the upper surfaces of materials due to the topology of the materials. These changes may then alter the water/surface contact characteristics that induce stronger adhesion of water to the metals as compared to astroquartz and graphite composite. The SEM images (Fig. 3) support this hypothesis and indicate that the metals had rougher surfaces than astroquartz and graphite composite materials.

These results support the conclusion that the drying processes of liquids on spacecraft materials might affect the aggregation of bacterial spores present in liquids, and, thus, might affect the survival of terrestrial microorganisms on sun-exposed surfaces of landed Mars spacecraft. Schuerger *et al*. (2003) had previously shown that the inactivation of endospores of *B. subtilis* HA101 progresses very quickly under simulated clear-sky conditions on Mars. In simulations within the same MEC system as used here (including a similar UV fluence rate and spectrum), endospores of *B. subtilis* HA101 were reduced by over five orders of magnitude after only 15 min under Mars-normal conditions of pressure, temperature, gas composition, and UV irradiation (Schuerger *et al*., 2003). However, if bacterial spores and vegetative cells can be concentrated into large multilayered colonies on external surfaces of spacecraft materials through the drying processes of contaminated liquids, then the results reported here suggest that microbial survival under martian UV irradiation might be enhanced significantly. This conclusion is supported by the work of Mancinelli and Klovstad (2000), who clearly demonstrated increased survival rates of *B. subtilis* HA101 in multilayered colonies under simulated martian UV irradiation.

However, microbial contamination on spacecraft can occur through the dry deposition of contaminated dusts (Venkateswaran *et al*., 2001; La Duc *et al*., 2003), and, thus, the results reported herein may not be applicable to situations in which liquids are avoided during microbial sampling or sanitizing procedures on spacecraft materials. For example, although dry deposition of contaminated dusts in payload processing facili-

ties is likely a primary means of contaminating spacecraft surfaces (Venkateswaran *et al*., 2001), many of the surfaces of assembled spacecraft are treated with a variety of liquid-associated assay procedures (Venkateswaran *et al*., 2001; La Duc *et al*., 2003, 2004). For areas of spacecraft that are cleaned or sampled with a liquid-associated assay, the results of the current study are likely to apply. Sampling and cleaning processes that include liquids may result in the formation of multilayered aggregates if the drying process is not controlled or the spacecraft material is suitable for the formation of large multilayered aggregates, as described herein. But not all areas of the spacecraft are cleaned or sampled with liquid-associated assays, and, thus, the areas that remain dry may avoid the formation of multilayered colonial aggregates. We seek to call out only the possibility that liquids interacting with some spacecraft surfaces may contribute to the formation of multilayered colonial aggregates of microbial spores. Under situations in which spacecraft surfaces remain dry throughout the payload processing phase of the mission, the UV-protective effects of multilayered colonies may not be a major factor.

Additional studies are warranted to investigate the effects of other spacecraft materials on the survival of terrestrial microorganisms under simulated martian conditions. Efforts should be made to identify other spacecraft materials that might promote the aggregation of microbial communities into multilayered colonies, and to determine how to best inactivate these aggregated spores prior to launch. In addition, long-term multi-sol UV exposures should be tested in order to determine whether the most buried cells in multilayered aggregates can be inactivated by longer exposures to UV irradiation on Mars. The longest UV exposure in the current study was for 1 h. Although a significant decrease was noted for the multilayered colonies on astroquartz and graphite composite under a 1-h UV irradiation, it is possible that UV exposures representing several sols on Mars might have completely inactivated the populations of *B. subtilis* on these materials.

Previous work has demonstrated that terrestrial bacteria on sun-exposed spacecraft surfaces on Mars are likely to be inactivated very quickly under clear sky conditions (Schuerger *et al*., 2003, 2004). This conclusion was based on the assumption that the bacteria were fully exposed to the UV irradiation on smooth horizontal surfaces. However, the complex topology of spacecraft materials may extend microbial survival on Mars by offering some protection from UV irradiation. Bacteria adhering to smooth horizontal surfaces or in shallow pits (Fig. 6A) are likely to be inactivated the fastest because the cells are exposed directly to both direct (solid lines in Figs. 6 and 7) and diffuse (dashed lines in Figs. 6 and 7) UV irradiation under most sky conditions. Under such circumstances, the rapid kill-rates as described by Schuerger *et al*. (2003) are likely to hold true. As the complexity and depth of the pits increase (Fig. 6B–E), the survival rates of embedded cells are likely to be longer than on horizontal surfaces. But as long as UV irradiation can freely penetrate down and into the complex cavities of the surface topology of spacecraft materials and directly contact cells, then embedded bacteria are likely to accumulate biocidal dosage levels of solar UV irradiation. For example, bacteria lodged at the bottoms of narrow pits (Fig. 6B) may still be inactivated by direct UV irradiation during a given sol on Mars because the direct UV irradiation at midday may still be adequate in both intensity and duration during the short time of exposure at midday. This is supported by the observation that although some endospores of *B. subtilis* on stainless steel surfaces in the current study were lodged within shallow vertical pits (Fig. 3), they were still inactivated by 1 h of UV irradiation (Fig. 2B). In addition, endospores of *B. subtilis* were observed in larger and more complex pits on uncoated aluminum-6061 (Fig. 3), but yet were fully inactivated after 1-h UV exposures. Thus, we propose that as long as UV photons are able to penetrate the shallow surface features of spacecraft materials, the embedded spores within these topological features may still accumulate biocidal dosage levels of UV irradiation rendering the populations inactive. Even if the pits are deep (Fig. 6C) or form overhangs in which UV photons must bounce off several surfaces before contacting viable cells (Fig. 6D), eventually microbial cells would accumulate biocidal dosages of UV irradiation. For example, Schuerger *et al*. (2003) report that a viable bioload of endospores of *B. subtilis* HA101 was reduced five orders of magnitude in 15 min under a clear-sky Mars UV simulation. If viable endospores of *B. subtilis* were present within a complex pit with an overhang that reduced the instantaneous UV

fluence rate to 1% of a fully exposed horizontal surface, then the embedded cells would require at least 1,500 min of solar irradiation (approximately 2 sols on Mars based on a 12-h/12-h day/night diurnal cycle) to achieve the same level of bioload reduction as observed on fully exposed horizontal surfaces. And lastly, both direct (solid lines) and diffuse (dashed lines) UV irradiations contribute to the inactivation of microbial cells on and within spacecraft surface features (Fig. 6E). It is therefore the total UV flux that is important in predicting microbial survival on Mars. Depending on sun angles at different latitudes or dust-load conditions of the atmosphere, there might be microbial bioloads on spacecraft surfaces that are exposed to both direct and diffuse UV irradiation, and then other scenarios in which microbial bioloads are exposed to only diffuse UV irradiation. Thus, regardless of the direct versus diffuse nature of the UV irradiation striking a spacecraft surface on Mars, as long as biologically effective UV photons (Cockell *et al*., 2000) at appropriately high energies (*i.e*., UVC photons between 200 and 280 nm) can penetrate down and into surface features on spacecraft materials, viable cells of terrestrial microorganisms would slowly accumulate lethal doses of UV irradiation. This process may lead to greater reductions in viable bioloads on spacecraft surfaces than originally thought possible.

These models are based on the assumptions that UV photons can freely penetrate down into the various cracks, crevices, and pits within spacecraft materials. However, if UV irradiation is attenuated or scattered by any barriers including (i) UV-absorbing compounds (Fig. 7A), (ii) bacteria present as multilayered microbial colonies within spacecraft pits and cracks (Fig. 7A), or (iii) UV irradiation blocked by spacecraft welds or secured joints (Fig. 7B), then the fully protected microbial bioloads would not be expected to be adversely affected by solar UV irradiation on Mars. More research is required to characterize the precise nature of microbial bioloads on spacecraft surfaces, and more research is required to characterize UV-absorbing materials on spacecraft that might coat microbial colonies and increase their survival on Mars.

CONCLUSIONS

The results presented herein support the conclusions by Schuerger *et al*. (2003) that bacteria present on sun-exposed spacecraft surfaces on Mars may be inactivated very rapidly under clear-sky conditions. We have extended the discussion of microbial survival on spacecraft surfaces on Mars to include complex surface topologies of spacecraft materials, and provided evidence that viable cells of spore-forming bacteria may be inactivated under Mars UV fluence rates even when embedded or lodged within cracks, crevices, and pits within the upper surfaces of spacecraft materials. We also demonstrated that multilayered aggregates of spores can form on the non-metal surfaces of astroquartz and graphite composite materials. Large multilayered colonial aggregates of viable microorganisms on certain spacecraft materials might lead to increased survival rates of bacteria transported to Mars by a process in which UV photons are absorbed by overlying layers of cells that leads to the protection of cells in underlying layers of the colonies. We recommend that future research: (a) characterize the actual forms and densities of microbial colonies on current spacecraft hardware, (b) determine what UV-absorbing materials might be used during payload processing activities that could cover microbial colonies on spacecraft surfaces (*e.g*., spacecraft lubricants, adhesives, kapton tape), and (c) determine if much longer UV simulations of martian conditions can sterilize microbial bioloads on spacecraft surfaces given the protective effects of multilayered microbial colonies discussed here.

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ABBREVIATIONS

MEC, Mars Electrostatic Chamber; MPN, Most Probable Numbers; NIR, near-infrared light; SDIW,

sterile deionized water; SEM, scanning electron microscopy; UV, ultraviolet; VIS, visible light.

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Address reprint requests to:
Andrew C. Schuerger
University of Florida
Building M6-1025
Space Life Sciences Lab
Kennedy Space Center, FL 32899

E-mail: acschuerger@ifas.ufl.edu